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Performance of Selective Media for Enumerating *Zygosaccharomyces bailii* in Acidic Foods and Beverages

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ABSTRACT

A study was undertaken to evaluate the performance of yeast-malt extract agar (YMA) (control medium) and three selective media, acidified YMA (AYMA), acidified tryptone-glucose-yeast extract agar (TGYA), and *Zygosaccharomyces bailii* selective agar (ZBA), for detecting and enumerating nine strains of *Z. bailii* grown in six commercial food products with a_w and pH values ranging from 0.82 to 0.99 and 2.99 to 6.47, respectively. These media were also evaluated for their suitability for enumerating two strains of *Z. bailii* grown in blueberry syrup containing 0, 300, and 600 μg of sodium benzoate ml^{-1} and subsequently stored at 1 and −19°C. The nonselective enumeration medium (YMA) supported significantly ($P \leq 0.05$) higher recovery of all strains of *Z. bailii* from the six food products compared to the three selective media; TGYA was the best selective medium, followed by ZBA. The performance of the selective media was dependent on the strain of *Z. bailii* and the food type. Recovery of cells of *Z. bailii* from blueberry syrup before storage or after storage at 1 or −19°C was equivalent on YMA, TGYA, and ZBA but inferior on AYMA, regardless of the benzoate concentration in the syrup in which the cells had grown. Cells in blueberry syrup held at −19°C exhibited a higher sensitivity to the acidic environment imposed by selective media compared to cells held at 1°C. Sensitivity to selective media was more apparent in cells grown in syrup containing no sodium benzoate compared to cells grown in syrup containing 300 or 600 μg of sodium benzoate ml^{-1}. It is recommended that TGYA be used for enumerating *Z. bailii* in acidic foods with reduced a_w, regardless of the presence of sodium benzoate in these foods or the reduced temperature at which they are held before being analyzed.

Key words: *Zygosaccharomyces bailii*, yeast, enumeration medium, acidic foods, cold stress

Zygosaccharomyces bailii is known to cause spoilage of acidic foods. Spoiled products may develop off flavors, off colors, turbidity, and sediment in brines and beverages, or a slimy or powdery film on solid surfaces of foods *(20, 26, 28, 31)*. High-sugar foods in which *Z. bailii* has grown may also have an ethanolic aroma as a result of fermentation *(16, 23)*. The yeast can grow over a temperature range of 6.5 to 37°C, depending upon the a_w of the substrate *(12)*. Yeasts normally do not play a major role in the spoilage of refrigerated or frozen foods due to their slow rate of growth and to low initial populations normally present in foods *(1, 27, 32)*. However, endemic problems associated with spoilage of fruit juices, soft drinks, salad dressings, and mayonnaise by *Z. bailii*, for example, are an ongoing concern to the food and beverage industry.

Benzoic and sorbic acids and their salts are sometimes used as preservatives in acidic foods and beverages. A reduction of the intracellular pH leads to an inhibition of metabolic processes, particularly those associated with uncoupling the active transport system necessary for energy supply *(14, 33)*. Some strains of *Z. bailii* are capable of growing in the presence of >600 μg of benzoic or sorbic acid ml^{-1} and have a remarkable tolerance to acid pH, being able to grow in media at pH as low as 2.2 *(22)*. Acetic acid is more toxic than lactic or citric acids *(31)*, but even 2% acetic acid does not prevent growth of some strains of *Z. bailii* in products such as tomato sauce *(22)*. Tolerance may be due in part to the ability of *Z. bailii* to metabolize these acids *(5, 10)* and to control permeability *(34)*.

Sugars are used as humectants to preserve some food products but differ in their effectiveness in controlling yeast growth *(15)*. *Z. bailii* is a moderately xerotolerant yeast *(28)*. Its ability to grow at reduced a_w is presumed to be due to the production of adaptive enzymes *(29)* or to shrinkage (dehydration) of cells, which can retard sugar uptake from the surrounding environment *(3, 24)*. The ability of xerotolerant yeasts to survive over a wide range of temperatures and other adverse environmental conditions at reduced a_w has been attributed to the intracellular accumulation of polyols *(4)*. Minimum a_w values for the growth of yeasts are influenced by the type of solute used to achieve reduced a_w as well as by pH, temperature, redox potential, and nutrient availability. The ability and state of adaptation to high concentrati ns of sugars can also influence the probability f growth on enumeration media with reduced a_w *(30)*.

Yeast cells stressed by high temperature require optimum recovery conditions to resuscitate *(8, 21)*. The extent and nature of injury caused by reduced temperature can vary depending upon the conditions under which the cells have been grown and the physical and chemical characteristics of

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the medium in which the cells are suspended. Tryptone-glucose-yeast extract (TGY) agar and malt-yeast extract agar (MEA) supplemented with 0.5% acetic acid have been recommended for detecting and enumerating preservative-resistant yeasts *(9)*. Erickson *(6)* developed *Zygosaccharomyces bailii* agar (ZBA) which selects for *Z. bailii* on the basis of the combined hurdle effects of acetic acid, potassium sorbate, and sodium chloride. The performance of these media for enumerating cells of *Z. bailii* stressed by reduced temperature and low a_w in acidic foods has not been assessed.

The objectives of this study were to determine the efficacy of nonselective yeast extract-malt extract agar (YMA) (control), acidified TGY (TGYA) agar, acidified YMA (AYMA) agar and ZBA agar for enumerating benzoate-resistant *Z. bailii* in six acidic foods and beverages. The ability of these media to support colony development by *Z. bailii* cells grown in blueberry syrup containing sodium benzoate and subsequently stored at 1°C or −19°C was also investigated.

MATERIALS AND METHODS

Strains and culture conditions for inocula

Source of strains. Nine strains of *Z. bailii* were used. Strains FRR 1299, FRR 2227, FRR 3671, FRR 3675, FRR 3680 and FRR 3705 originated from spoiled commercially processed foods and were kindly supplied by Dr. Ailsa D. Hocking, CSIRO Food Research Laboratory, New South Wales, Australia; strains 36946, 36947 and 14C 4806 were stock cultures of unknown origin. Cultures were maintained at 1°C on YMA (pH 6.2) slants prepared from YM broth (Difco Laboratories, Detroit, MI) supplemented with 1.5% agar. Yeasts were activated in YM broth (pH 6.2) at 30°C and transferred at 48-h intervals using loop inocula.

Adaptation to reduced pH. Acidified YM (AYM) broth (pH 4.0) was prepared by adding 17.4 N glacial acetic acid to sterile YM broth. Loop inocula of 48-h *Z. bailii* cultures (strains FRR 2227 and FRR 3680) grown in YM broth at 30°C were transferred to 10 ml of AYM broth in 16 by 125 mm test tubes. Subsequent loop transfers to AYM broth were made at 48-h intervals.

Adaptation to benzoate and reduced a_w. A mixture of commercial blueberry syrup (J. M. Smucker Co., Orrville, OH) and sterile deionized water (100:40, vol:vol, blueberry syrup:water; a_w 0.91) was supplemented with 0, 300, and 600 μg of sodium benzoate (USP, Pfizer Inc., New York, NY) ml^{-1} to give pH 3.36, 3.53, and 3.72, respectively. One milliliter of a 48-h AYM broth culture of *Z. bailii* grown at 30°C was transferred to 10 ml of control and benzoate-supplemented blueberry syrup in 20 by 125 mm test tubes. Inoculated syrups were incubated at 30°C and transfers (1.0 ml) were made to syrups with like concentrations of benzoate at 5-day intervals.

Enumeration media

Yeast extract-malt extract agar was used as a nonselective control medium for enumerating *Z. bailii*. The performance of three media formulated to select for yeasts resistant to benzoate, sorbate and/or other organic acids was evaluated. Acidified yeast extract-malt extract agar (AYMA, pH 3.8) was prepared by adding 5.0 ml of glacial acetic acid (17.4 N) to 1 liter of sterile YMA (47 to 52°C) before pouring it into petri plates. Acidified tryptone-glucose-yeast extract agar (TGYA, pH 3.9) contained 5.0 g of tryptone (Difco), 100 g of glucose (monohydrate), 5.0 g of yeast extract (Difco), 15 g of agar and 1 liter of deionized water. The sterilized, molten (47 to 52°C) medium was acidified by adding glacial acetic acid (17.4 N) (5.0 $ml/liter^{-1}$) before pouring it into petri plates. *Zygosaccharomyces bailii* selective agar (ZBA, pH 4.0) *(6)* was modified to contain (per liter of deionized water) 65 g of Sabouraud dextrose agar (Difco), 30 g of fructose, 25 g of sodium chloride, 5.0 g of tryptone, 2.5 g of yeast extract, 0.25 g of trypan blue, 0.10 g of potassium sorbate, and 5.0 ml of glacial acetic acid. The published formula *(6)* specifies using 30 g of Sabouraud dextrose agar but did in fact contain 65 g of Sabouraud agar. One milliliter of a sterile 10% solution of potassium sorbate and 5.0 ml of acetic acid (17.4 N) were added to the heated (121°C for 5 min), cooled (47 to 52°C) ZBA basal medium just before pouring it into petri plates. All media were prepared 2 to 5 days before use and held at 5°C. Plate temperature was adjusted to ca. 22°C during a 4 to 6 h period immediately before test samples were applied.

Recovery of Z. bailii *from foods and beverages*

The ability of media to allow recovery of cells of nine strains of *Z. bailii* grown in six commercial foods and beverages was investigated (Table 1). Test strains cultured in YM broth for 5 days at 30°C were individually inoculated (1.0 ml) into 100 g of each food or beverage. Inoculated products were thoroughly mixed and then incubated at 25°C for 21 days. Cultures used as inocula were serially diluted (1:10) in sterile 0.1% peptone solution and surface-plated (0.1 ml) in duplicate on YMA agar to determine initial populations in each product. Plates were incubated at 30°C for 5 days before colonies were counted.

After 21 days of incubation at 25°C, foods were analyzed for populations of *Z. bailii*. Duplicate 10-g samples of each thoroughly mixed product were combined with 90 ml of sterile 30% glucose solution, mixed, serially diluted and surface plated (0.1 ml) in duplicate on YMA, AYMA, TGYA, and ZBA. Colonies were counted after incubation at 30°C for 5 days.

Recovery of cold-stressed Z. bailii *from blueberry syrup*

YMA, AYMA, TGYA, and ZBA were evaluated for their suitability to resuscitate and support colony development by cold-stressed cells of *Z. bailii*. Two *Z. bailii* strains (FRR 2227 and FRR 3680) were adapted to benzoate by culturing at 30°C in diluted blueberry syrup (a_w 0.91) containing 0, 300, or 600 μg of the preservative ml^{-1}. One milliliter of a 5-day culture was serially diluted in sterile 30% glucose solution and surface plated (0.1 ml) in duplicate on YMA and the three selective media. Inoculated blueberry syrup incubated for 5 days at 30°C and then stored for 21 days at 1°C and −19°C was also analyzed for populations of *Z. bailii*. Samples were warmed by holding at 23°C for approximately 10 min, mixed, serially diluted in sterile 30% glucose solution and surface plated (0.1 ml) in duplicate on the four enumeration media.

TABLE 1. *Water activity, pH values and type of preservative used in commercial food products*

Food	a_w	pH	Preservative
Sweetened condensed milk	0.84	6.47	None
Chocolate syrup	0.83	5.14	Potassium sorbate
Date pastry filling	0.86	4.67	Citric acid and sodium benzoate
Carbonated beverage	0.99	3.31	Citric acid and sodium benzoate
Blueberry syrup	0.82	3.28	Citric acid
Wine	0.99	2.99	Alcohol

Plates were incubated at 30°C and colonies were counted after 5 days.

Statistical analysis

Two or more replicates of each experiment were done in duplicate. Data were analyzed using the general linear model procedure of the Statistical Analysis Systems (SAS) procedure (SAS Institute, Cary, NC) to test for statistically significant differences ($P \leq 0.05$). Mean separation of values was obtained by the least squares difference method.

RESULTS AND DISCUSSION

Foods selected for evaluation differed in composition, a_w, pH, and the presence and type of preservative (Table 1). Populations of *Z. bailii* recovered from inoculated foods on YMA and three selective enumeration media are listed in Table 2. Strains FRR 1299, FRR 2227, 36947, and 14C 4806 grew in all six products, whereas FRR 3675, FRR 3680, and 36946 grew in at least three of the six foods and beverages. Strains FRR 3671 and FRR 3705 failed to grow in any of the test foods within the 21-day incubation period at 25°C. The performance of media in allowing the recovery of *Z. bailii* varied, depending upon the test strain and type of food. Populations of strains FRR 3680 and 36946 recovered from date filling were significantly ($P \leq 0.05$) lower on YMA than on selective TGYA or ZBA. Significantly higher populations of strain 14C 4806 were recovered from chocolate syrup and wine on AYMA and ZBA, respectively, than on YMA. Otherwise, YMA usually supported significantly higher recovery of *Z. bailii* compared to the three selective media. Skidmore and Koburger *(25)* attributed the high pH of rose bengal-tetracycline medium *(2)*, in comparison with acidified PDA, to its ability to recover high populations of yeasts. When the latter medium was adjusted to below pH 5.0, its ability to recover yeasts and molds from a variety of processed foods was reduced considerably *(17)*. Table 3

TABLE 2. *Mean populations of seven* Z. bailii *strains recovered from six foods and beverages on yeast-malt extract agar (YMA) and three selective media*

			Z. bailii (log CFU ml^{-1} [g^{-1}])[a]					
			Food/beverage[b]					
Strain[c]	Enumeration medium[d]	Initial population	Condensed milk	Date filling	Chocolate syrup	Blueberry syrup	Wine	Carbonated beverage
FRR 1299	YMA	5.85	1.95 a	5.82 a	1.48 a	4.23 a	4.56 a	3.63 a
	AYMA		1.09 b	5.72 b	0.85 b	3.33 d	4.13 a	3.61 a
	TGYA		1.68 a	5.83 a	1.44 a	3.93 b	4.61 a	3.67 a
	ZBA		0.85 b	5.74 b	1.45 a	3.84 c	4.56 a	3.63 a
FRR 2227	YMA	4.91	1.50 a	5.43 a	1.60 a	2.45 a	4.59 a	3.83 a
	AYMA		1.30 a	5.20 c	0.85 ab	0.35 c	4.48 b	3.44 d
	TGYA		1.65 a	5.34 b	1.63 a	2.47 a	4.52 ab	3.75 b
	ZBA		0.70 b	5.23 c	0.59 b	1.53 b	4.47 b	3.61 c
FRR 3675	YMA	4.82	0.50 ab	5.34 a	1.45 a	—[e]	—	—
	AYMA		0.35 b	4.57 b	1.30 a	—	—	—
	TGYA		0.67 ab	5.37 a	1.20 a	—	—	—
	ZBA		0.85 a	5.37 a	0.35 b	—	—	—
FRR 3680	YMA	4.85	1.54 a	4.76 b	0.85 a	—	0.35 a	2.00 a
	AYMA		0.47 d	4.53 b	—	—	—	1.66 bc
	TGYA		1.29 b	5.23 a	—	—	0.35 a	1.93 ab
	ZBA		0.85 c	5.21 a	—	—	0.50 a	1.64 c
36946	YMA	4.74	—	5.48 b	—	—	0.35 a	1.15 a
	AYMA		—	5.43 b	—	—	—	0.85 b
	TGYA		—	5.67 a	—	—	—	0.85 b
	ZBA		—	5.61 a	—		—	0.35 c
36947	YMA	5.33	2.30 a	5.71 a	1.00 a	1.87 a	1.69 a	2.56 a
	AYMA		1.57 c	5.11 c	0.85 a	—	1.39 a	2.51 a
	TGYA		2.27 a	5.63 b	—	1.08 ab	1.41 a	2.20 a
	ZBA		1.68 ab	5.57 b	—	1.58 ab	1.27 a	1.41 b
14 C 4806	YMA	4.68	2.32 a	5.41 a	—	2.91 a	3.25 b	3.75 ab
	AYMA		1.59 c	5.07 b	0.73 a	0.59 c	3.38 b	3.84 c
	TGYA		1.91 bc	5.42 a	0.15 b	2.77 a	3.41 b	3.70 b
	ZBA		1.09 d	5.35 a	0.15 b	2.26 b	3.52 a	3.41 c

[a] Values within the same strain and food that are not followed by the same letter are significantly different ($P \leq 0.05$).
[b] Inoculated foods were stored at 25°C for 21 days before analysis.
[c] Two strains tested (FRR 3671 and FRR 3705) did not grow in test foods within 21 days at 25°C.
[d] YMA, yeast-malt extract agar; AYMA, acidified YMA; TGYA, tryptone-glucose-yeast extract agar; ZBA, *Z. bailii* selective agar.
[e] Less than 1 CFU ml^{-1} (g^{-1}).

TABLE 3. *Composite mean populations of seven strains of* Z. bailii *recovered from six foods and beverages on YMA and three selective media*

Enumeration medium	*Z. bailii* (log CFU ml^{-1} [g^{-1}])[a] Food/beverage[b]					
	Condensed milk	Date filling	Chocolate syrup	Blueberry syrup	Wine	Carbonated beverage
YMA	1.44 a	5.42 a	0.91 a	1.64 a	2.11 a	3.44 a
AYMA	0.93 b	5.09 b	0.65 b	0.61 b	1.91 a	2.27 a
TGYA	1.36 a	5.50 a	0.63 ab	1.46 a	2.04 a	2.30 a
ZBA	0.86 b	5.44 a	0.36 b	1.32 ab	2.06 a	2.01 a

[a] Values within the same column that are not followed by the same letter are significantly different ($P \leq 0.05$). The mean initial population was 5.03 log CFU ml^{-1} (g^{-1}).
[b] Inoculated foods and beverages were stored at 25°C for 21 days before analysis.

presents mean populations of *Z. bailii* recovered from six foods and beverages on YMA and three acidified selective media. The order of performance was YMA = TGYA > ZBA > AYMA. The AYMA medium was inferior for recovering *Z. bailii* from products with reduced a_w.

YMA and selective media were compared for recovering *Z. bailii* strains FRR 2227 and FRR 3680 grown in blueberry syrup containing sodium benzoate (0, 300, or 600 μg ml^{-1}) that was subsequently held for 21 days at 1°C or −19°C. Results are presented in Table 4. Regardless of the sodium benzoate concentration in blueberry syrup, recovery of control (0-day samples) or cold-stressed (21 days at 1°C) cells was as high on TGYA and ZBA as on YMA. Overall, recovery on AYMA was inferior.

Cells in some samples held at −19°C exhibited increased sensitivity to the acidic environment imposed by selective media. This was probably due to an inability of freeze-injured cells to resuscitate under the acidic pH conditions. MacLeod and Calcott *(18)* demonstrated that the freeze damage of the cells results in leakage of small molecular weight solutes. The greater sensitivity to acid pH values of some physically stressed yeast cells is one reason mycological media that depend upon broad-spectrum antibiotics for supression of bacteria give higher counts for fungi in some foods than do media acidified to pH 3.5 *(13, 17)*.

Sensitivity of cells held at −19°C to the acidic pH of selective media was more evident when cells had been grown in blueberry syrup containing no benzoate compared to sensitivity of cells grown in syrup containing 300 or 600 μg of benzoate ml^{-1}. Cells held at 1°C were less sensitive to selective conditions of enumeration media compared to cells held at −19°C. This was probably due to the greater ability of cold-stressed cells to tolerate the acidity of selective media compared to freeze-injured cells.

Jermini et al. *(11)* reported that *Z. rouxii* and *Torulaspora delbrueckii* grew in yeast extract-glucose (YEG) broth

TABLE 4. *Mean populations of* Zygosaccharomyces bailii *grown in blueberry syrup containing 0, 300, and 600 μg of sodium benzoate ml^{-1}, stored at 1 and −19°C for 21 days and plated on YMA and three selective media*

Benzoate (μg ml^{-1})	Enumeration medium[b]	Population (log CFU ml^{-1})[a] Strain FRR 2227				Strain FRR 3680			
		1°C		−19°C		1°C		−19°C	
		0 d	21 d	0 d	21 d	0 d	21 d	0 d	21 d
0	YMA	4.77 ab	4.40 a	4.90 a	3.43 a	4.68 a	4.66 a	4.77 a	4.49 a
	AYMA	4.58 b	4.46 a	4.62 a	2.91 b	4.45 b	4.47 a	4.60 b	4.27 b
	TGYA	4.80 a	4.81 a	4.86 a	3.10 b	4.75 a	4.55 a	4.82 a	4.38 ab
	ZBA	4.79 a	4.67 a	4.76 a	3.09 b	4.76 a	4.68 a	4.75 a	4.37 ab
300	YMA	4.16 a	4.21 a	4.41 a	3.97 a	4.54 ab	4.54 a	4.52 a	4.23 a
	AYMA	3.99 b	3.93 b	4.33 a	3.45 b	4.45 b	4.37 b	4.42 a	3.99 a
	TGYA	4.15 a	4.19 a	4.43 a	3.77 ab	4.57 a	4.56 a	4.58 a	4.11 a
	ZBA	4.17 a	4.27 a	4.48 a	3.78 ab	4.56 ab	4.61 a	4.57 a	4.14 a
600	YMA	4.15 a	4.10 b	4.17 a	3.50 a	4.16 a	4.12 b	4.15 a	3.53 a
	AYMA	3.88 b	3.89 c	4.03 b	3.37 a	4.13 a	4.09 b	4.10 a	3.33 a
	TGYA	4.21 a	4.20 ab	4.13 ab	2.93 a	4.24 a	4.16 ab	4.20 a	4.43 a
	ZBA	4.31 a	4.23 a	4.22 a	3.40 a	4.24 a	4.20 a	4.15 a	3.41 a

[a] Values in the same column within each benzoate concentration in blueberry syrup in which *Z. bailii* was grown and stored that are not followed by the same letter are significantly ($P \leq 0.05$) different.
[b] YMA, yeast-malt extract agar; AYMA, acidified YMA; TGYA, tryptone-glucose-yeast extract agar; ZBA, *Z. bailii* selective agar.

containing 10% glucose at 4°C but *Z. bailii* did not. None of these yeasts grew at 4°C in YEG broth containing 50% glucose. Survival of microorganisms is greatly affected by the composition of the medium in which they are frozen *(19)*. Ingredients such as sugars, sodium chloride and other nutrients can have a protective effect on yeast cells. In this study, sugar undoubtedly enhanced the ability of *Z. bailii* to survive in blueberry syrup stored at −19°C and 1°C. Golden and Beuchat *(7)* demonstrated that glucose and sucrose protected against heat- and freeze-inactivation of *Z. rouxii* cells, regardless of the presence of potassium sorbate in media used to culture the organism before low-temperature treatment. *Z. rouxii* was tolerant to freezing at −18°C for up to 120 days in media supplemented with glucose, sucrose, or NaCl.

It is recommended that TGYA be used as a selective medium for enumerating cold- or freeze-stressed cells of *Z. bailii* from acidic and reduced a_w foods. However, caution must be taken when interpreting results of analysis using TGYA to select and enumerate freeze-stressed cells of *Z. bailii* and perhaps other preservative-resistant yeasts, since not all viable cells can resuscitate in the acidic environment imposed by this and other selective media. There is still a need for a medium that can select for all *Z. bailii* in acid-preserved foods and beverages, particularly when cells have been stressed by reduced temperature or a_w.

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